Original article

Natural killer cells contribute to hepatic injury and help in viral persistence during progression of hepatitis B e-antigen-negative chronic hepatitis B virus infection

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ABSTRACT

Hepatitis B e-antigen negative (e−) chronic HBV infection (CHI) encompasses a heterogeneous clinical spectrum ranging from inactive carrier (IC) state to e− chronic hepatitis B (CHB), cirrhosis and hepatic decompensation. In the backdrop of dysfunctional virus-specific T cells, natural killer (NK) cells are emerging as innate effectors in CHI. We characterized CD3+ CD56− NK cells in clinically well-defined, treatment-naive e− patients in IC, e−CHB or decompenated liver cirrhosis (LC) phase to appraise their role in disease progression. The NK cell frequencies increased progressively with disease severity (IC 8.2%, e−CHB 13.2% and LC 14.4%). Higher proportion of NK cells from LC/e−CHB expressed CD69, Nkp46, Nkp44, TRAIL and perforin, the last two being prominent features of CD56bright and CD56dim NK subsets, respectively. The frequencies of CD3+ CD56− NK cells together with TRAIL+ CD56bright and Perforin+ CD56dim NK cells correlated positively with serum alanine transaminase levels in e−CHB/LC. K562 cell-stimulated NK cells from e−CHB/LC exhibited significantly greater degranulation but diminished interferon-γ production than IC. Further, Perforin− NK cell frequency inversely correlated with autologous CD4+ T-cell count in e− patients and ligands of NK receptors were over-expressed in CD4+ T cells from e−CHB/LC relative to IC. Co-culture of sorted CD56dim NK cells and CD4+ T cells from e−CHB showed enhanced CD4+ T-cell apoptosis, which was reduced by perforin inhibitor, concanamycin A, suggesting a possible perforin-dependent NK cell-mediated CD4+ T-cell depletion. Moreover, greater incidence of perforin-expressing NK cells and decline in CD4+ T cells were noticed intrahepatically in e−CHB than IC. Collectively, NK cells contribute to the progression of e−CHI by enhanced TRAIL- and perforin-dependent cytolytic activity and by restraining anti-viral immunity through reduced interferon-γ secretion and perforin-mediated CD4+ T-cell lysis. S. Ghosh, CMI 2016;22:733.e9–733.e19

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Introduction

The outcome of hepatitis B virus (HBV) infection and the pathogenesis of associated liver diseases are believed to be determined by host–virus interactions mediated by the immune response [1]. During the course of chronic HBV infection (CHI), the loss of hepatitis B e-antigen (HBeAg) and development of anti-HBe are known to confer a favourable outcome in patients and coincide with normalization of liver function, loss/decrease of serum HBV DNA, subsidence of hepatic inflammation and clinical remission [2]. Patients with such quiescent infection are referred to as ‘inactive carriers’ (IC) [2]. However, active hepatitis may develop in about one-third of IC without reversion of HBeAg in their serum [2]. This phase called HBeAg-negative (e−) chronic hepatitis B (CHB), is currently the most predominant and aggressive type of CHB and its sequel may include advanced fibrosis, cirrhosis, hepatic...
decompensation or even hepatocellular carcinoma [2]. Liver damage during HBV infection has conventionally been attributed to cytolytic killing of infected hepatocytes by virus-specific CD8+ T cells [3]. However, different studies have demonstrated that these cells are markedly depleted and functionally impaired in patients with CHB [3], thereby suggesting important roles of other immune cells in contributing to liver damage. Natural killer (NK) cells are a major constituent of the lymphocyte-rich hepatic inflammatory infiltrate found in patients with CHB [3]. Characterized by the absence of T-cell receptor/CD3 complex and expression of CD56, NK cells represent 5–10% of the peripheral and ~30% of the intrahepatic lymphocyte pools in humans [4]. Of the two key NK cell functions, the cytolytic activity is mostly confined to CD56bright NK cells, predominant in peripheral blood whereas the release of antiviral cytokines, interferon-γ (IFN-γ) and tumour necrosis factor-α has been attributed to CD56dim NK cells that are enriched in tissues [4]. NK cell activation is mediated through its activating-receptors (including NKG2D, NKG2C, Nkp44, Nkp46) that bind to their cognate ligands expressed on the surfaces of target cells [3]. They induce target-cell apoptosis by either directed secretion of cytolytic granules containing perforin and granzyme or through signalling via death receptor–ligand pathways such as TRAIL/FasL [3]. Further, there is growing evidence that NK cells can also exhibit immunoregulatory functions and can limit T-cell immunity [5].

Studies have tried to comprehend the role of NK cells in acute HBV infection and in distinct phases or episodes in CH, with a major focus on HBeAg-positive CHB [6–9] and have yielded, in part, conflicting results. In this study we investigated the frequency, phenotype and functions of NK cells and their subsets in treatment-naïve Indian patients representing different phases of e(−)CHI to gain a clearer understanding of their contribution towards disease progression and pathogenesis that may well translate into strategies for effective management of these patients.

Materials and methods

Study participants and samples

Consecutive, treatment-naïve individuals (n = 88) with e(−)CHI were enrolled from the Hepatology clinic of the School of Digestive and Liver Diseases, Institute of Post Graduate Medical Education and Research, Kolkata, India between 2011 and 2014. Virological, biochemical and clinical assessments were performed before recruitment that were necessary to precisely define the e(−)CHI cohort into IC, e(−)CHB and decompensated liver cirrhosis (LC) subgroups. Exclusion criteria included co-infection with human immunodeficiency virus/hepatitis C virus/hepatitis D virus, significant co-morbidities like diabetes mellitus, chronic alcoholism, intravenous drug abuse or evidence of any overt infection such as spontaneous bacterial peritonitis, urinary tract infection or pneumonia in the recent past. IC included participants with HBV DNA <10⁶ copies/mL and alanine transaminase (ALT) <40 IU/L, both measured on three occasions 2 months apart, with no biochemical/radiological/clinical/histological evidence of liver disease. The e(−)CHB patients were characterized by HBV DNA >10⁶ copies/mL, ALT >40 IU/L, histological activity index >3 and absence of cirrhosis. LC was diagnosed by a combination of clinical/biochemical/radiological/endoscopic features [10] and signs of liver decompensation (ascites/variceal bleeding/hepatic encephalopathy/non-obstructive jaundice). Child–Pugh score was calculated in LC to determine severity of liver disease [10]. Additionally, HBV-uninfected healthy individuals were included as healthy controls (HC). The study was approved by Institutional Ethics Committee and each participant provided written informed consent before enrolment. Blood samples were drawn in EDTA tubes from all participants and liver biopsy specimens were obtained from selected IC and e(−)CHB patients.

Reagents and chemicals

Fluorochrome-conjugated anti-human monoclonal antibodies, immunological solutions/buffers and other biochemical reagents were purchased from BioLegend (San Diego, CA, USA), BD Biosciences (San Jose, CA, USA), BD Pharmingen (San Diego, CA, USA) and Sigma Aldrich (St Louis, MO, USA) respectively.

Frequency and phenotype of NK cells and CD4+ T cells

To assess the frequency/subset/phenotype of NK cells and percentage of MHC class I polyopeptide-related sequence A (MICA)/MICA-expressing CD4+ T cells, blood samples were incubated with anti-CD3-peridinin chlorophyll protein (PerCP), anti-CD56-allophycocyanin (APC), anti-CD69-fluorescein isothioyionate (FITC), anti-NKp46-FITC, anti-NKGD2-phycoerythrin (PE), anti-NKp44-PE, anti-TRAIL-PE, anti-CD4–FITC and anti-MICA/MICB–APC in appropriate combinations and treated with BD FACS Lysing Solution. After washing, stained cells were analysed using flow cytometry.

Intracellular staining of the lytic proteins

To detect intracellular perforin/granzyme-B in NK cells, peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood using Histopaque density gradient centrifugation and stained with anti-CD3-PerCP and anti-CD56–FITC. After fixation and permeabilization using a BD Cytotox/Cytoperm kit, cells were stained with anti-Perforin–APC or anti-Granzyme-B–AlexaFluor 647 followed by fluorescence-activated cell sorting (FACS) analysis.

Degranulation of NK cells and IFN-γ production

The PBMCs of e(−) patients were stimulated with K562 cells at an effector-to-target ratio of 10:1. Anti-CD107a–FITC and monensin (6 mg/L) were added directly to the medium 2 h later. After another 3 h of incubation at 37°C in 5% CO₂, the cells were harvested, washed and stained with anti-CD3-PerCP and anti-CD56–APC and CD107a+ CD3− CD56− NK cell frequency was evaluated by flow cytometry. Unstimulated PBMCs served as negative controls.

To determine the percentage of IFN-γ+ NK cells, PBMCs were co-cultured with K562 cells for 6 h. Brefeldin A (10 mg/L) was added 2 h post-stimulation. Subsequently, cells were stained with anti-CD3-PerCP and anti-CD56–FITC, fixed, permeabilized and stained intracellularly with anti-IFN-γ–APC.

CD4+ T-cell True count

For determining the absolute counts of CD3+ CD4+ T cells, blood samples of e(−) patients were processed using BD CD3/CD8/CD45/CD4 Multitest reagent and Trucount tubes (BD Biosciences) and acquired on a flow cytometer.

Expression analysis of ligands for NK receptors on sorted CD4+ T cells

From freshly isolated PBMCs of different e(−) patients and HC, CD4+ T cells were magnetically sorted (purity >97%) using CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Total RNA was isolated from CD4+ T cells by TRIzol RNA extraction principle and cDNA was generated by a Reverse Transcription kit (Life Technologies,
Carlsbad, CA, USA) and used to quantify the expression of NKG2D-ligand (MICA) and that of the cellular ligand of Nkp44 (Nkp44L) by SYBR green Real-Time PCR with gene-specific primers (see Supplementary material, Table S1). Each sample was assayed in triplicate and 18S rRNA was used as the internal control for relative quantification of gene expression.

Co-culture of sorted NK cells and CD4\(^+\) T cells for cytotoxicity assay

Autologous CD56\(^{dim}\) NK cells and CD4\(^+\) T cells were sorted (purity >97%) from fresh PBMCs of e(−)CHB patients using a CD56\(^++\) CD16\(^-\) NK cell isolation kit and CD4 MicroBeads (Miltenyi Biotec) and were stimulated with 50 U/mL of recombinant human interleukin-2 (rhIL-2) overnight in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics. NK cells and CD4\(^+\) T cells were then mixed at ratio of 10:1 in 96-well plates and co-cultured for 24 h at 37\(^\circ\)C.

To evaluate perforin-mediated cytotoxicity, NK cells were incubated with 2.5 nM concanamycin A (Sigma Aldrich) for 90 min before co-culture. CD4\(^+\) T cells cultured alone were used as controls. After co-culture, lymphocytes were harvested, stained with anti-human-CD4-APC and frequency of apoptotic CD4\(^+\) T cells was determined using an FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) following a manufacturer-guided protocol. Cells positive for only Annexin V but not propidium iodide were considered as early apoptotic events whereas those that stained positive for both dyes were labelled as late apoptotic events.

Flow cytometry

For all analysis, viable lymphocytes were gated based on their forward and side-scatter characteristics followed by fluorescence. NK cells were defined as CD3\(^-\) CD56\(^{dim}\) lymphocytes and were classified into CD56\(^{dim}\) and CD56\(^{bright}\) subtypes according to CD56 fluorescence intensity (Fig. 1a). Within these populations, the frequency of cells having a particular phenotype was noted for each sample. Isotype/unstimulated controls were used as appropriate in all assays. At least 8000 events per run in NK-gate were acquired in each experiment whereas a total of 30 000 events were acquired for cytotoxicity assay. Data were acquired and analysed using FACS CALIBUR and CellQuest PRO Software or FACS VERSE and FACS SUITE software (BD Biosciences).

Immunohistochemical and haematoxylin & eosin staining

Immunohistochemistry was performed on 6-\(\mu\)m paraffin-embedded liver biopsy tissue sections of selected IC and e(−)CHB patients. After deparaffinization, hydration and antigen retrieval [1], sections were incubated overnight at 4\(^\circ\)C with either a combination of anti-Nkp46-FITC and anti-Perforin-APC or only anti-CD4-FITC in 1 : 1000 dilution. The slides were then washed in PBS containing 0.1% Tween-20, cover-mounted with ProLong Gold antifade reagent (Life Technologies) and examined under confocal microscope (Leica Microsystems, Wetzlar, Hesse, Germany). Haematoxylin & eosin staining of tissue sections was performed for evaluation of liver histological status of the patients.

Statistical analysis

Data were analysed using GRAPHPAD PRISM5 software. Comparisons between groups were carried out using one-way analysis of variance followed by Tukey's Multiple Comparison Test or Kruskal–Wallis test followed by Dunn's Multiple Comparison Test as appropriate. Spearman's rank correlation test was used for correlation analysis. For all tests, \(p < 0.05\) was considered statistically significant.

**Results**

**Clinical, serological and demographic data**

Based on clinical, biochemical, serological, virological and histological assessments, 88 HBeAg-negative patients were categorized as IC (\(n = 30\)), e(−)CHB (\(n = 38\)) and LC (\(n = 20\)) (see Supplementary material, Table S2). All LC patients had decompensated liver disease of Child–Pugh grade B. In addition, 20 HC were included.

**Increased frequency of peripheral NK cells during progressive phases of e(−)CHB**

Phenotypic analysis of PBMCs from enrolled patients demonstrated that the proportion of circulating CD3\(^-\) CD56\(^-\) NK cells was significantly expanded in LC (mean 14.4 ± 1.4%) and e(−)CHB patients (mean 13.2 ± 1.1%) compared with IC (mean 8.2 ± 0.4%) and HC (mean 8.8 ± 0.8%) (Fig. 1a,b). The distribution of both CD3\(^-\) CD56\(^{dim}\) and CD3\(^-\) CD56\(^{bright}\) NK subtypes also showed a similar increasing trend with disease progression with CD56\(^{dim}\) NK cells being most abundant peripherally.

**Increased activation of NK cells in patients with active liver disease**

CD69 is one of the earliest activation markers expressed on activated NK cells [11]. Although IC and HC showed no difference in incidence of CD69\(^+\) NK cells, frequency of such cells was significantly up-regulated in LC and e(−)CHB (Fig. 1c,d), indicating that the activated status of NK cells is related to disease severity. We further investigated the expression of prominent activation receptors on NK cells (Nkp46, Nkpg2D and Nkp44) that are important for NK cell triggering. As illustrated in Fig. 1e, NK cells expressing Nkp46 and Nkp44 were significantly high in LC and e(−)CHB relative to IC and HC, but no variation in frequency of Nkp2D\(^+\) NK cells could be perceived in any groups.

**Up-regulation of TRAIL on NK cells in diseased patients**

The interaction between TRAIL (expressed by NK cells) and its receptor (on target cells) constitutes an important apoptotic pathway leading to liver injury. We noted significant increase in frequency of TRAIL\(^+\) NK cells in advancing clinical phases of e(−)CHB, with the majority of TRAIL noted on the CD56\(^{bright}\) subset. CD56\(^{bright}\) NK cells from LC displayed the highest levels of TRAIL (mean 65.2 ± 2.9%), followed by e(−)CHB (mean 57.8 ± 3.8%), both of which were significantly higher than IC (mean 45.1 ± 2.9%) and HC (mean 36.7 ± 4.8%) (Fig. 2a,b), suggesting an important role of this pathway in the pathogenesis of HBV-induced liver disease.

**Enhanced perforin and granzyme expression with disease severity**

NK cells also use the granule–exocytosis pathway for target cell lysis and we examined the frequencies of perforin/granzyme-B\(^+\) NK cells peripherally in different categories of e(−) patients. Unlike TRAIL, both perforin and granzyme-B were predominantly expressed by the CD56\(^{dim}\) subset. We observed an increasing trend in the frequency of Perforin\(^+\) CD56\(^{dim}\) NK cells among IC (mean 38.6 ± 3.9%), e(−)CHB (mean 59.3 ± 3.5%) and LC (mean 75.6 ± 2.7%) with the differences in frequencies being significant in each pairwise comparison (Fig. 2a,c). However, granzyme-B was almost constitutively expressed by most CD56\(^{dim}\) NK cells and no statistical
Fig. 1. Frequency of activated natural killer (NK) cells increased with disease severity in hepatitis B e-antigen (HBeAg)-negative chronic hepatitis B virus infection. (a) Representative flow cytometric dot plots from healthy controls (HC), inactive carriers (IC), patients with HBeAg-negative chronic hepatitis B (CHB) and patients with decompensated liver cirrhosis (LC) indicating percentages of CD3⁺CD56⁺, CD3⁺CD56dim and CD3⁺CD56bright NK cells among lymphocytes. (b) Cumulative data of frequencies of NK cells and their subsets in different study participants. (c) Representative FACS dot plots depicting frequencies of gated CD3⁺CD56⁺ NK cells expressing CD69 among various study groups. (d) Bar diagram showing percentages of NK cells expressing CD69 in different study groups. (e) Pooled data exhibiting frequencies of NK cells expressing activation receptors NKp46, NKG2D and NKp44 in HC, IC, CHB and LC. ***p < 0.0001, **p < 0.001 and *p < 0.05.
Fig. 2. Natural killer (NK) cells skewed towards cytotoxicity during progression of hepatitis B e-antigen (HBeAg)-negative chronic hepatitis B virus infection. (a) Representative FACS dot plots depict frequencies of gated CD3+CD56+ NK cells expressing TRAIL, perforin and granzyme-B among healthy controls (HC), inactive carriers (IC), patients with HBeAg-negative chronic hepatitis B (CHB) and patients with decompensated liver cirrhosis (LC). Grouped bar diagram demonstrating pooled data of percentages of NK cells and their subsets expressing (b) TRAIL, (c) perforin and (d) granzyme-B. Correlation analysis between serum alanine transaminase (ALT) levels versus (e) percentages of CD3+CD56+ NK cells or (f) TRAIL+CD3+CD56bright NK cells or (g) Perforin+CD3+CD56dim NK cells among HBeAg-negative patients. ***p < 0.0001, **p < 0.001, *p < 0.05.
difference in granzyme-B⁺ CD56dim NK cells could be perceived among different e(−) groups. Surprisingly, CD56bright NK cells showed significantly high levels of granzyme-B in LC/e(−)CHB compared with IC/HC (Fig. 2a,d), albeit at levels lower than that produced by the CD56dim subset in all cases.

Direct ex vivo correlation between frequency of cytotoxic NK cells and HBV-related liver injury

Serum ALT is a widely used surrogate marker for liver damage [12]. The e(−)CHB and LC patients included in this study displayed elevated ALT levels (see Supplementary material, Table S2) and also carried increased percentages of NK cells expressing potent cytotoxic mediators, TRAIL and Perforin. Given these findings, we investigated if there was an associated increase in the cytotoxic potential of NK cells and the corresponding serum ALT levels. Spearman’s correlation analysis revealed a significant positive correlation between ALT levels and the percentage of CD3⁻ CD56⁺ NK cells in patients with active hepatitis (r = 0.6656, p < 0.0001) (Fig. 2e). Furthermore, TRAIL and Perforin expressions on bright and dim NK cell subsets, respectively, were found to strongly associate with raised serum ALT values in the active HBV carriers (Fig. 2f,g). These data indicated that both TRAIL as well as perforin might be closely involved in inducing significant collateral hepatic tissue injury in patients with ongoing liver necroinflammation.

Skewing of NK cells towards degranulation in progressive phases of e(−)CHI

A hallmark of NK cell activation is degranulation, i.e. release of lytic granule contents, perforin and granzymes, onto the surface of target cells. To explore the cytotoxic ability of NK cells from e(−) patients, we used a flow cytometric degranulation assay that measures the mobilization and surface expression of CD107a/LAMP1 on NK cells after interaction with K562 target cells [13]. Although a minor fraction of ex vivo NK cells from IC (mean 10.04 ± 1.9%) and HC (mean 6.9 ± 1.2%) degranulated upon stimulation with K562 target cells, a significant increase in the frequency of CD107a⁺ NK cells was observed in LC (mean 21.2 ± 3.1%) and e(−) CHB (mean 21.6 ± 2.2%) (Fig. 3a,b), demonstrating the hyper-cytolytic potential of NK cells with disease severity. As expected, the CD56dim subpopulation displayed stronger cytotoxicity,
Natural killer (NK) cells restrain CD4<sup>+</sup> T-cell response in hepatitis B e-antigen (HBeAg)-negative patients with active liver disease. (a) Correlation analysis between absolute CD4<sup>+</sup> T-cell count and perforin<sup>+</sup> NK cells in HBeAg-negative patients (Spearman $r = -0.5831$, p value (two-tailed) 0.01). Messenger RNA expression patterns of (b) NKp44-ligand (NKp44L) and (c) NKG2D-ligand MICA in sorted CD4<sup>+</sup> T cells derived from healthy controls (HC), inactive carriers (IC) and patients with active disease that included both HBeAg-negative chronic hepatitis B (CHB) and decompensated liver cirrhosis (LC) were determined by real-time RT-PCR. Bar diagram (d) and representative flow cytometry dot plots (e) exhibiting percentages of MICA/MICB-expressing CD4<sup>+</sup> T cells in different study participants. (f) A representative FACS analysis profile of a cytotoxicity assay demonstrating increased apoptosis of autologous CD4<sup>+</sup> T cells upon co-culture with CD56<sup>dim</sup> NK cells sorted from a CHB patient and a relative reduction in apoptotic rate following pre-incubation of NK cells with 2.5 nM of concanamycin A before co-culture. Upon co-culture, CD4<sup>+</sup> T cells were observed to have predominantly undergone late apoptosis as determined by marked increase in dual (Annexin V and propidium iodide) positive events as shown in the upper right quadrant of the representative dot plots. *p < 0.05, **p < 0.005.
although CD56^{bright} cells also degranulated, albeit to a much lesser extent.

**Reduced IFN-γ production by NK cells**

We evaluated the capacity of NK cells in different groups of e(−) patients to produce IFN-γ, post-stimulation by K562 cells. As judged by the percentages of IFN-γ + NK cells, LC (mean 2.7 ± 0.5%) and e(−)CHB (mean 3.6 ± 0.3%) were significantly impaired in their IFN-γ-producing ability in comparison with IC (mean 6.3 ± 1%) and HC (mean 4.4 ± 0.6%) (Fig. 3c,d). When compared between NK cell subsets, intriguingly, CD56^{dim} NK cells were found to be the major cytokine producers relative to the CD56^{bright} subset (Fig. 3c).

**Correlation between absolute CD4^+ T-cell count and Perforin^+ NK cells**

Given that perforin/granzyme-expressing NK cells are abundant in e(−)CHB/LC and since CD4^+ T cells were far more susceptible to direct killing by NK cells than CD8^+ T cells [5], we speculated that NK cells could predominantly target and lyse CD4^+ T cells in a perforin-dependent manner in the setting of e(−)CHI. We investigated the relationship between frequency of perforin^+ NK cells and absolute count of autologous CD4^+ T cells of IC (n = 6), e(−)CHB (n = 8) and LC (n = 4). A significant negative correlation was observed between frequencies of CD4^+ T cells and perforin^+ NK cells (Fig. 4a; r = −0.5831, p < 0.01), suggesting a perforin-dependent depletion of CD4^+ T cells.

**Over-expression of NK-receptor ligands in CD4^+ T cells of diseased patients**

To test whether increased sensitivity of CD4^+ T cells of e(−)CHB/LC to NK lysis was due to increased expression of ligands for activating NK receptors, we compared the expression of mRNA encoding NK22D-ligand (MICA) and Nkp44-ligand (Nkp44L) by real-time PCR in CD4^+ T cells sorted from five HC, five IC and six patients with active liver disease including both e(−)CHB and LC. Compared with IC/HC, a significant up-regulation of both Nkp44L and MICA transcripts was perceived in CD4^+ T cells of e(−)CHB/LC (Fig. 4b,c). Similarly, a significantly higher frequency of CD4^+ T cells expressing MICA/MICB protein was observed in e(−)CHB/LC patients relative to IC/HC (Fig. 4d,e), thereby making these CD4^+ T cells an effective target for NK-cell-mediated lysis through the NKG2D/Nkp44-induced perforin-pathway.

**CD56^{dim} NK cells exhibit cytotoxicity against autologous CD4^+ T cells**

A cytotoxicity assay was performed by co-culturing purified, autologous and IL-2 pre-activated CD56^{dim} NK cells and CD4^+ T cells from e(−)CHB patients to demonstrate a rapid elimination of CD4^+ T cells by NK cells. In the representative experiment shown in Fig. 4f, CD4^+ T cells when cultured alone showed 7.67% apoptotic events of which 4.46% were positive for only Annexin V, i.e. early apoptotic, and 3.21% were dual positive for both Annexin V and PI, i.e. late apoptotic. However, co-culture of activated NK cells and CD4^+ T cells depicted a sharp increase in apoptotic events (27.26%) with the majority of the affected cells having undergone late apoptosis (23.53%) whereas a fraction (3.73%) showed signs of early apoptosis. Interestingly, significant target cell death occurred at low effector : target ratios (i.e. 10 : 1), indicating effective cytotoxic potential of activated NK cells. Moreover, addition of even a low dose of 2.5 nM concanamycin A reduced the cytotoxicity of target CD4^+ T-cells (Fig. 4f), thereby suggesting the process to be perforin-dependent.

**Accumulation of Perforin^+ NK cells and decline in CD4^+ T cells in livers of patients with active disease**

To evaluate the intrahepatic incidence of NK cells in HBeAg-negative patients, we studied the frequency of Nkp46^+ cells as well their expression of perforin in liver biopsy sections of five e(−)CHB and five IC patients by immunohistochemical staining since Nkp46 is considered to be the most specific marker for NK cells [14]. Liver histology indicated that lymphocyte-predominant lobular and portal inflammation was more prominent in e(−)CHB than IC (Fig. 5a). Nkp46^+ cell density was found to increase dramatically in the liver of e(−)CHB compared with IC (Fig. 5b). Moreover, Nkp46^+ NK cells in e(−)CHB patients were found to be enriched in perforin whereas in IC, a low number of Nkp46^+ perforin^+ NK cells were observed. Further, confocal imaging depicted the presence of fewer liver resident CD4^+ T cells in e(−)CHB than IC (Fig. 5c). Taken together, the data suggest that the increased prevalence of Perforin^+ NK cells might have potentiated CD4^+ T-cell depletion within the intrahepatic compartment.

**Discussion**

Previous studies on NK cells in viral hepatitis B had revealed in many cases divergent data, with one study reporting a temporal relationship between ALT flares and increased circulating and intrahepatically activated NK cells in CHB [11], whereas another failed to detect any increase in activated NK cells during flares [7]. Moreover, while Zhang et al. [9] documented increased cytolytic activity of intrahepatic NK cells during HBeAg-positive CHB, Li et al. [8] showed deficient NK effector function in patients with analogous clinical conditions. The evolving clinical landscape of e(−)CHI provides an excellent opportunity to delineate the role of NK cells in immunopathology during its different phases that vary in severity. Given that none of the chronically HBV-infected study participants had other viral/bacterial infections, the induction of the innate immune response was mostly related to HBV infection.

The highest frequency of NK cells was exhibited by LC followed by e(−)CHB and IC and strongly correlated with ALT levels, a marker of liver injury. An array of activating and inhibitory receptors have been implicated in NK cell activity [15]. A significant up-regulation of activation receptors (namely, Nkp30, Nkp44, Nkp46 and NKG2D) and reduced expression of the inhibitory receptors CD158a and CD158b were observed on NK cells in CHB patients in different studies [6,9]. In contrast, low expression of Nkp30 and NKG2D on NK cells had also been reported in patients with similar disease profiles [8]. Given that we found a progressive increase in the proportion of CD69-expressing NK cells with disease severity, and as there exists a direct correlation between surface density of activating receptors and magnitude of cytolytic activity of NK cells [16], we focused primarily on the frequencies of NK cells bearing various activating receptors in different phases of e(−)CHI. Although no variation was noted in the frequency of NKG2D^+ NK cells in different study groups, a significant expansion of NK cells expressing Nkp46 and Nkp44 was consistently observed in e(−)CHB and LC, relative to IC/HC. Hence our findings were in concordance with previous studies that advocate for a strong association between NK cell activation and disease pathogenesis.

The differences in activating NK cell receptor expression in different categories of HBeAg-negative patients were also mirrored by clear functional disparity of NK cells from these groups. The TRAIL^+ as well as Perforin^+ NK cells were found to be significantly amplified in LC and e(−)CHB patients, contrasting with minimal
Fig. 5. Increased prevalence of Perforin expressing NKp46⁺ natural killer (NK) cells with a concomitant drop in CD4⁺ T cells intrahepatically with disease severity. (a) Haematoxylin & eosin staining of liver biopsy sections in patients with hepatitis B e-antigen (HBeAg) -negative chronic hepatitis B (CHB) and inactive carriers (IC). (b) Confocal immunofluorescence images showing NKp46⁺ NK cells (upper panel), their intracellular perforin content (middle panel) and their co-localization (lower panel) in CHB and IC. (c) Immunohistochemical detection of CD4⁺ T cells in liver tissues of CHB and IC.
had been documented that NK cells could mediate perforin-mediated killing of target cells. Further, a higher prevalence of intrahepatic NK cells and up-regulation of perforin was found in e−/CHB, in comparison with IC, implying an involvement of this cytolytic pathway in disease pathogenesis. It had been documented that NK cells are among the earliest arrivals at the site of inflammation and generally reside in hepatic sinusoids. Although both CD56dim and CD56bright NK cells migrate to inflamed liver, the CD56dim subset is preferentially recruited during active necro-inflammation, suggesting that the highly cytotoxic CD56dim cells may be the key players exacerbating liver injury in the setting of e−/CHI. However, a relative resistance of virally infected hepatocytes to perforin/granzyme-mediated killing had also been reported. In addition to direct cytotoxicity, NK cells also produce IFN-γ, important in the control of viral infections, and this function had been depicted to be impaired in CHI [15,20,21]. We also noted a profound loss of NK cell-derived IFN-γ secretion by LC followed by e−/CHB, thereby making them most ineffective at exerting non-cytolytic antiviral functions. Mechanisms responsible for impaired IFN-γ secretion have not yet been clarified although recent evidence attributes a significant influence of co-inhibitory molecule Tim-3 [22], IL-10 [21] in causing this NK cell defect. We also found that LC patients presented with the highest serum IL-10 levels compared with other groups (data not shown), which may suppress the ability of NK cells to produce IFN-γ in these patients. Although cytokine production was mainly assigned to CD56bright NK cells, we noted appreciable levels of IFN-γ production by CD56dim NK subsets at 6 h after NK cell stimulation. Our results were consistent with the findings of De Maria et al. [23] who described CD56dim cells as early producers of IFN-γ upon receptor-mediated stimulation.

It is being increasingly recognized that NK cells can regulate T-cell responses and this is particularly strong under conditions of viral infections [5,24]. The NK cell-mediated regulation of CD8+ T-cell responses in patients with chronic HBV had been addressed by a study in which HBV-specific CD8+ T cells were shown to up-regulate TRAIL receptors that rendered them susceptible to apoptosis by TRAIL [25]. Moreover, it had been recently reported that in patients treated with nucleos(t)ide analogue, depletion of NK cells or TRAIL/NKG2D blockade resulted in significant improvement of HBV-specific T-cell responses, particularly the CD4+ T-cell function [24]. We observed an enormous amplification in the proportion of Perforin+ NK cells in e−/CHB/LC and it had been documented that NK cells could mediate perforin-dependent restriction of both CD8+ T and CD4+ T-cell responses [5]. However, CD4+ and CD8+ T cells differ in their susceptibility to NK cell lysis, with NK cells having a greater propensity to lyse CD8+ T cells than CD8+ T cells that expressed high levels of CD48, which engages the inhibitory receptor CD244 on NK cells [5]. Hence, we speculated a possible interaction between NK cells and CD4+ T cells in e−/CHI that could result in the gradual loss of CD4+ T cells and eventually influence disease chronicity. Our observation of a strong inverse correlation between percentages of circulating CD4+ T cells and perforin+ NK cells in e−/CHI lends credence to this hypothesis. This negative association remained significant even when LC were excluded from analysis (data not shown), given that cirrhosis of the liver could induce splenic sequestration of peripheral T cells, resulting in diminished CD4+ T-cell counts [26]. Moreover, a decline in intrahepatic CD4+ T cells during active disease further supports our hypothesis that NK cell cytotoxicity might have attributed to CD4+ T-cell depletion. To gain an insight into the molecular mechanism of CD4+ T-cell suppression by NK cells, we asked whether differential expression of NK-receptor ligands in CD4+ T cells of various categories of e− patients contributed to the differences in NK sensitivity. We demonstrated increased expression of the natural ligands of NKG2D and Nkp44 in CD4+ T cells from e−/CHB/LC patients relative to IC, which may be responsible for triggering NK cells to kill CD4+ T cells via NK receptor—ligand interactions and subsequent granule exocytosis, although the underlying factors and signalling that promote ligand expression in these T cells remain to be investigated. Previous research indicated that CD4+ T lymphocytes infected with human immunodeficiency virus 1 also expressed Nkp44 [27] as well as NKG2D ligands ULBP1-3/3 but not MICA/MICB [28] that were able to stimulate the cytotoxic response of NK cells. It had been demonstrated that IFN-γ can down-regulate the expression of MICA [29] and might represent a regulatory mechanism to prevent strong sustained triggering of the NKG2D receptor. Given the observed deficiency in NK cell IFN-γ production in e+/CHB/LC, it seems plausible that the cytokine milieu in these patients might influence the heightened expression of NKG2D ligands in the CD4+ T cells and consequently their increased recognition by NK cells. Finally by in vitro co-culture of sorted CD56dim NK cells and autologous CD4+ T cells from e− CHB patients, we showed enhanced annexin reactivity of CD4+ T cells in the presence of NK cells and a decrease in apoptotic rate following treatment with perforin-inhibitor, concanamycin A, suggesting perforin-dependent elimination of CD4+ T cells by NK cells. Our findings thereby extended the functional reach of the NK system in CHI to include NK-mediated negative regulation of CD4+ T-cell responses. We put forward a previously unappreciated perforin-dependent immunoregulatory role of NK cells, particularly by CD56dim subset, on CD4+ T cells during progressive phases of e−/CHI. Removal of CD4+ T cells from the system eliminates help for CD8+ T cells, which results in CD8+ T-cell exhaustion along with inadequate humoral immune response [5], allowing HBV to persist within host during e−/CHI. Our study showed that during e−/CHI, enhanced NK cell cytotoxicity accompanied by insufficient IFN-γ production and NK-mediated, perforin-dependent depletion of CD4+ T cells perpetuated liver damage as well as maintained HBV persistence, which in turn contributed to the development of advanced liver diseases. However, a caveat in this study was its cross-sectional character. A longitudinal analysis of sequential changes in NK cell activity starting from IC to the advance of the disease would have been more meaningful for assessing the impact of NK cells on progression of e−/CHI. But such prospective studies are not feasible due to ethical implications and official guidelines prompting early initiation of anti-viral therapy for e−/CHB patients. Our findings that NK cells may enhance the immunopathology associated with e−/CHI brings an interesting aspect to HBV therapy. It had been demonstrated that IFN-α can inhibit virus replication but can also activate the NK cells [30]. Hence a regimen that combines IFN-α and therapeutic depletion of NK cells or blockade of NK-activating receptors might be more successful in HBV eradication.

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Appendix A. Supplementary data

Additional Supporting Information may be found in the online version of this article at http://dx.doi.org/10.1016/j.cmi.2016.05.009.

Transparency Declaration

The authors have no conflicts of interest to disclose in connection with the submitted manuscript.

References